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ORIGINAL ARTICLE

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p53-independent G_1 cell cycle arrest of human colon carcinoma cells HT-29 by sulforaphane is associated with induction of $p21^{CIP1}$ and inhibition of expression of cyclin D1

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Abstract Isothiocyanate sulforaphane (SFN) is a potent cancer chemopreventive agent. We investigated the mechanisms underlying the anti-proliferative effects of SFN in the human colon carcinoma cell line, HT-29. We demonstrate that SFN inhibits the growth of HT-29 cells in a dose- and time-dependent manner. Treatment of serum-stimulated HT-29 cells with SFN suppressed the re-initiation of cell cycle by inducing a G_1 phase cell cycle arrest. At high doses (>25 μM), SFN dramatically induces the expression of $p21^{CIP1}$ while significantly inhibits the expression of the G_1 phase cell cycle regulatory genes such as cyclin D1, cyclin A, and c-myc. This regulation can be detected at both the mRNA and protein levels as early as 4 h post-treatment of SFN at 50 μM. Additionally, SFN activates MAPKs pathways, including ERK, JNK and p38. Exposure of HT-29 cells with both SFN and an antioxidant, either NAC or GSH, completely blocked the SFN-mediated activation of these MAPK signaling cascades, regulation of cyclin D1and $p21^{CIP1}$ gene expression, and G_1phase cell cycle arrest. This finding suggests that SFN-induced oxidative stress plays a role in these observed effects. Furthermore, the activation of the ERK and p38 pathways by SFN is involved in the upregulation of $p21^{CIP1}$ and cyclin D1, whereas the activation of the JNK pathway plays a contradictory role and may be partially involved in the downregulation of cyclin D1. Because cyclin D1 and $p21^{CIP1}$ play opposing roles in G_1 phase cell cycle progression regulation, blocking the activation of each MAPK pathway with specific MAPK inhibitors, is unable to rescue the SFN-induced G_1 phase cell cycle arrest in HT-29 cells.

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Keywords Sulforaphane · Cell cycle arrest · HT-29 cells · Mitogen-activated protein kinase

Abbreviations SFN: Sulforaphane · MAPK: Mitogen-activated protein kinase · NF-κB: Nuclear factor-kappa B · GSH: Glutathione · NAC: *N*-acetyl-L-cysteine · MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt · ERK: Extracellular signal-regulated protein kinase JNK, c-Jun NH_2-terminal kinase

Introduction

Colon cancer is a major cause of cancer-associated mortality in the US [1]. Epidemiologic studies indicate that the incidence of colon cancer is inversely correlated with the consumption of fruits and vegetables [2]. Therefore, the use of cancer chemopreventive agents derived from dietary or other natural sources to block or slow the onset of premalignant tumors, such as colon carcinomas, has been widely accepted as a strategy for decreasing the incidence of colon cancer. In the search for fruit or vegetable phytochemicals with anti-carcinogenic effects, isothiocyanates have gained much attention. Isothiocyanates are not naturally present in cruciferous vegetables, such as broccoli and cauliflower, but are generated from secondary metabolites known as glucosinolates during the process of vegetable crushing or chewing [3]. Also, they may be produced in the intestines where micorflora can promote the hydrolysis of glucosinolates to isothiocyanates [4]. Sulforaphane (SFN), one of the major isothiocyanates, has potent anti-carcinogenic activity in rodent carcinogenesis models of lung [5], mammary gland [6, 7], stomach [8], colon [9, 10], and bladder [11] cancers. The mechanism(s) by which SFN exerts its anti-carcinogenic effect(s) are not fully understood. However, it is known that SFN can induce the

metabolism of carcinogens, thereby blocking their ability to initiate the development of cancer. This can be achieved in two, non-mutually exclusive ways. First, SFN, through its regulation of Nrf2-mediated gene transcription [12, 13], can strongly induce phase II detoxification enzymes, such as glutathione S-transferase, epoxide hydrolase, quinone reductase and UDP-glucuronolsyltransferase. This induction would allow for the accelerated excretion of pro-carcinogens or activated carcinogens prior to cancer initiation. Second, SFN can inhibit some phase I drug metabolism enzymes [14, 15]. Inhibition of these enzymes, such as cytochrome P450 enzymes, which are involved in carcinogen activation, may play a role in the ability of SFN to block cancer initiation.

More recently, in vitro studies have suggested some additional anti-cancer properties of SFN. These include the activation of mitogen-activated protein kinases (MAPKs) pathways [16], the generation of mild oxidative stress [17], and the regulation of NF-κB signaling pathways [18]. Because each of these mechanisms are involved in inducing cell cycle arrest and apoptosis [19–22], SFN also may act as a cancer suppressor that directly halts or retards the growth of established cancers [23, 24].

Cyclins are key cell cycle control molecules with specific, periodic expression associated with cell cycle progression [25]. Other cell cycle control molecules include cyclin-dependent kinase (cdk) inhibitors, such as $p21^{CIP1}$ and $p27^{KIP1}$, which tightly regulate the activities of cyclin/CDK enzyme complexes [26]. Mitogen-activated protein kinases, which are activated in response to a wide variety of extracellular stimuli, mediate signal transduction cascades that play important roles in cell proliferation, differentiation, cell cycle control, and apoptosis [27]. Recent studies indicate that MAPKs also are involved in the regulation of cyclin D1 expression [28] and p53-independent $p21^{CIP1}$ induction [29]. Our interest on the study of regulation of cell cycle control by SFN in HT-29 cells stemmed from one of our primary studies in which we found that SFN and another isothiocyanate, phenylethyl isothiocyanate (PEITC), can strongly inhibit the expression of a cyclinD1-luciferase reporter gene in transiently transfected HT-29 cells (unpublished data). Because cyclin D1 is the rate-limiting component in promoting G_1 phase progression, the inhibition of cyclin D1 transcription by SFN suggests that SFN may induce G_1 phase cell cycle arrest. And previous studies have shown that SFN could induce cell cycle arrest in HT-29 cells [19, 30]. Therefore, inhibition of cyclin D1 expression could be a potential mechanism of growth inhibition by SFN. The present study examines the molecular mechanism by which SFN causes inhibition of cell growth in human colon carcinoma cells, with specific focus on regulation of cell cycle control-related cyclin D1 and $p21^{CIP1}$ gene expression, as well as the role of MAPKs activation induced by SFN in those cellular events.

Materials and methods

Cell culture and reagents

Human colorectal cancer cells, HT-29, which contain a mutated p53 gene, were purchased from American Type Culture Collection (ATCC). HT-29 cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2.2 g/l sodium bicarbonate, 100 U/ml penicillin, and 100 mg/ml streptomycin. SFN was purchased from LKT lab. SB203580 (p38 inhibitor), SP500125 (JNK inhibitor) and PD98059 (ERK inhibitor) were purchased from Calbiochem (La Jolla, CA, USA).

Cell viability assay (MTS assay)

Cells were cultured in 24-well plates at a density of 1×10^4 cells/well. Twenty-four hours later, cells were serum starved for 36 h, then cells were treated with SFN (dissolved in DMSO) at different doses or vehicle (DMSO) for additional 12 h or 24 h. Cell viability was determined by using the CellTiter non-radioactive cell proliferation assay kit (Promega, Madison, WI, USA).

Flow cytometric analysis of cell cycle distribution

HT-29 cells were seeded in 60-mm petri-dishes at density of 5000 cell/cm^2 in complete MEM medium overnight. The cells were then serum-starved for 36 h in serum-free MEM medium, which was then replaced with complete MEM medium containing either 0.1 % DMSO (as a negative control) or varying doses of SFN (6.25, 12.5, 25, 50 and 100 μM) for 12 h or 24 h. Additionally, a time course using a treatment of 50 μM SFN was performed similarly. Following SFN treatment, the cells were collected by trypsinization and processed for flow cytometric analysis. Briefly, collected cells were washed twice in ice-cold PBS and collected by centrifugation at 2500 rpm for 5 min. The cell pellet was then re-suspended in 500 μl of PBS, and the resulting cell suspension was passed through a 26.5 gauge needle 3 times. The cells were then fixed using 3 ml of ice-cold 70% ethanol added while vortexing. Fixed cells were placed on ice for 30 min, then collected by centrifugation and washed again with 0.5 ml of PBS. The fixed cell pellets were then stained in 0.5-ml PBS containing 100 μg/ml RNase A and 10 μg/ml propidium iodide at 4°C in the dark overnight. Cell cycle distribution was then analyzed by flow cytometry using FACS analysis core facility of University of Medicine and Dentistry of New Jersey.

Reverse-transcriptase and polymerase chain reaction (RT-PCR)

HT-29 cells were plated in 100-mm petri-dishes and serum-starved for 36 h. Cells were cultured again in

complete MEM medium and treated with SFN. Following treatment, total RNA was isolated by using an RNeasy Mini kit (Qiagen). First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript First-strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. PCR reactions were performed by using 1 μl of RT product, 1 μl of primer mixture (final concentration 10 μM) and 23 μl of Invitrogen Platinum Super Mixture. Primers to specifically amplify the genes interested were as follows: forward, 5′-ATT AGT TT ACC TGG ACC CAG-3′ and reverse, 5′-GAT GGA GCC GTC GGT GTA GAT GCA-3′ for cyclin D1 gene; forward, 5′-GTG AGC GAT GGA ACT TCG A-3′ and reverse, 5′-AAT CTG TCA TGC TGG TCT GC-3′ for p21 gene; forward, 5′-ATT AGT TTA CCT GGA CCC AG-3′ and reverse, 5′-CAC AAA CTC TGC TAC TTC TG-3′ for cyclin A gene; forward, 5′-AGT TCT CGG CTC GCT CCA GGA AGA-3′ and reverse, 5′-TCT TGT GTC GCC ATA TAC CGG TCA-3′ for cyclin E; forward, 5′-CAA GAG GCG AAC ACA CAA CGT CT-3′ and reverse, 5′-AAC TGT TCT CGT CGT TTC CGC AA-3′ for c-myc gene. For the internal control gene actin, forward, 5′-CGT ACC ACT GGC ATC GTG AT-3′ and reverse, 5′-GTG TTG GCG TAC AGG TCT TTG-3′ were used. Eight microliters of the amplified products was run on a 1.0% agarose gel followed by visualization using ethidium bromide staining.

Western blot analysis

After treatment, HT-29 cells in six-well plates were washed with ice-cold PBS and lysed with 200 μl of whole cell lyses buffer (10 mM Tris–HCl, pH 7.9, 250 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.5% Triton X-100, 10% glycerol, 1 × proteinase inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride, 100 μM Na_3VO_4, 5 μM $ZnCl_2$, 2 mM indole acetic acid). After one freeze-thaw cycle at −80°C, the cell lysates were centrifuged at 12,000 *g* for 10 min at 4°C. The protein concentrations of the whole cell lysate supernatants were determined using a Bio-Rad protein assay kit. An equal amount of protein (20 μg) was then resolved on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane using semi-dry transfer system. The membrane was blocked in 5% non-fat milk for 1 h at room temperature, then incubated overnight at 4°C with a primary antibody specifically recognizing cyclin D1 (sc-718), $p21^{CIP1}$ (sc-6246), cyclin A (sc-596), or actin (sc-1616) (Santa Cruz Biotechnology). After incubation with the primary antibody, the membrane was washed with TBST (20 mM Tris–HCl, 8 g/l NaCl, 0.1% Tween 20, pH 7.6) three times, then incubated with horseradish peroxidase-conjugated secondary antibody (1:5000 dilution) for 45 min at room temperature followed by an additional three washes with TBST. Detection was performed using ECL reagents (Bio-Rad). To detect phosphorylation, HT-29 cells grown in six-well plates were lysed in 200 μl of kinase lysis buffer (10 mM Tris–HCl, pH 7.4, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 mM sodium orthovandate, 2 mM iodoacetic acid, 5 mM zinc chloride, 1 mM phenylmethylsulfonyl fluoride and 0.5% Triton X-100). Following SDS-PAGE and protein transfer (as described above), the membrane was blocked by 5% bovine serum albumin solution. Antibodies to detect specific phosphorylated residues were as follows: ERK1/2 (Thr 202/Tyr 204), JNK (Thr 183/Tyr 185), p38 (Thr 180/Tyr 182) (1:2000 dilutions, Cell Signaling Technology). The pan ERK1/2 antibody was purchased from Santa Cruz Biotechnology; all the other general antibodies were products from Cell Signaling Technology.

Statistical analysis

Cell cycle distribution was analyzed by Student's *t* test. The results were considered statistically significant if P was less than 0.05.

Results

Sulforaphane inhibits cell growth and serum-stimulated reinitiation of cell cycle in HT-29 cells

After serum starvation for 36 h, the growth inhibition effects of SFN on serum-stimulated HT-29 cells' growth were analyzed by MTS assay. As shown in Fig. 1, growth inhibition of HT-29 cells was detectable following 12 h or 24 h treatment using SFN concentrations starting from 25 μM. SFN inhibited serum-stimulated HT-29 cell growth in a very similar pattern at 12 h and 24 h; thus, 30–40% of growth inhibition was achieved at concentrations of 50 and 100 μM. Interestingly, cell growth was slightly promoted at low concentration of 12.5 μM. The observation that the growth inhibitory effects of high concentrations was slightly higher at 24 h than that of 12 h suggested that SFN induced a quick and sustained inhibition in serum-stimulated HT-29 cells growth.

In our previous studies, we found that SFN exhibits a dose- and time-dependent inhibition of expression of a cyclin D1-luciferase reporter gene transiently transfected into HT-29 cells (data not shown). Because cyclin D1 plays a key role in G_1 phase cell cycle regulation, and SFN could inhibit quiescent HT-29 cells to re-enter the cell cyle [30], flow cytometry analysis was used to confirm the cell cycle arrest effect of SFN in serum-stimulated HT-29 cells. For this analysis, HT-29 cells were synchronized at G_0/G_1 phase by serum-starvation for 36 h. The cells were then grown in medium containing 10% FBS and varying concentrations of SFN (or 0.1% DMSO as a negative control). From our results, compared to the DMSO-treated control cells, HT-29 cells

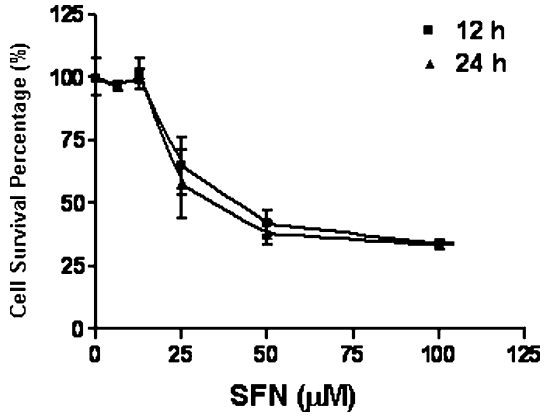


Fig. 1 Growth inhibitory effects of SFN on HT-29 cells. HT-29 cells were maintained in 24-well plates at a density of 1.0×10^4 cells/well. Cells were serum-starved for 36 h and then treated with SFN at dose of 6.25, 12.5, 25, 50 and 100 μM for additional 12 and 24 h. The MTS assay was performed as described in "Methods". The percentage of viable cell was calculated as a ratio of A_{490nm} of treated cells versus control cells (treated with 0.1% DMSO vehicle). Data presented are mean ± SD of two independent experiments

treated with 12.5, 25, 50 and 100 μM SFN for 12 h showed a significant ($P < 0.05$) increase in the percentage of G_1 phase cells accompanied by a decrease in the percentage of S phase cells. This effect was dose dependent. Additionally, following 24 h of 25 and 50 μM SFN treatment, a 10–20% increase of G_1 phase was also observed. It is interesting to note that treatment with 100 μM of SFN for 24 h has no G_1 arrest effect; however, the high percentage (47.49%) of sub-G_0 cells suggests a strong cytotoxicity associated with this high dose. The apparent loss of the G_1 arrest at low doses (12.5 and 25 μM) of SFN after 24 h suggests that the SFN-mediated effect is dose dependent, and that the intracellular concentration of SFN may decrease during long exposures due to metabolism or efflux. In a time-course study using 50 μM SFN, for control cells, the percentage of cells in G_1 phase decreases and the percentage of cells in S phase increases with time until 20 h. This effect is due to the growth stimulation induced by serum. At each time point analyzed, 50 μM SFN treatment strongly arrested cells in G_1 phase of the cell cycle with the maximum effect (81.59% of G_1 phase in treated cells vs. 41.20% of G_1 phase in control cells) at 20 h.

SFN alters the expression of G_1 cell cycle regulators

Because SFN induces a G_1 phase cell cycle arrest, we next examined whether SFN treatment alters the expression of key G_1 cell cycle regulatory genes such as cyclin D1, cyclin A, cyclin E, c-myc, $p21^{CIP1}$, and $p27^{KIP1}$ using semi-quantitative RT-PCR. After serum-starvation for 36 h, HT-29 cells were treated with 6.25, 12.5, 25, 50 or 100 μM SFN for 12 h in MEM containing 10% FBS. Total RNA was extracted from these cells and subjected to RT-PCR analysis to examine the cellular mRNA levels of the above-mentioned genes. As shown in Fig. 2a, cells treated with 50 and 100 μM SFN for 12 h display a marked decrease in cyclin D1, cyclin A, and c-myc gene expression. The expression of cyclin E did not significantly change following 12 h of treatment with any SFN concentration tested. In contrast, the expression of the cell cycle inhibitor gene $p21^{CIP1}$ was strongly induced by high concentration (> 25 μM) of SFN following 12 h of treatment with the highest induction observed at 50 μM. Surprisingly, the expression of another G_1 cell cycle related inhibitor gene $p27^{KIP1}$ did not show any change in expression level following SFN treatment in the same experiment (data not shown). To further examine the relationship between the altered gene expression and the cell cycle arrest described above, the transcription levels of the genes at six time points (4, 8, 12, 16, 20 and 24 h) following 50 μM

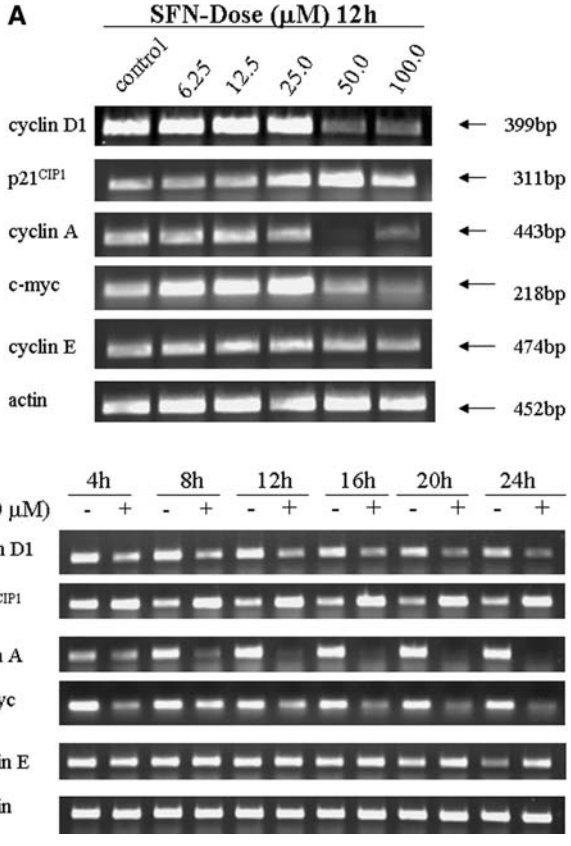


Fig. 2 Alterations of G_1 cell cycle regulatory gene expression by SFN. **a** HT-29 cells were serum-starved for 36 h. After changing to complete MEM medium and exposure to different doses of SFN (6.25, 12.5, 25, 50 and 100 μM) for 12 h, the mRNA levels of cyclin D1, $p21^{CIP1}$, cyclin A, cyclin E, and c-myc genes were detected semi-quantitatively by RT-PCR. Actin was served as an internal control. **b** HT-29 cells were serum-starved for 36 h. The cells were then grown in complete MEM medium and exposed to 50 μM SFN. Cells treated with SFN or a vehicle control were harvested at different time points (4, 8, 12, 16, 20 and 24 h). Total RNA was isolated and RT-PCR was performed to determine the mRNA levels of cyclin D1, $p21^{CIP1}$, cyclin A, cyclin E, and c-myc genes. Actin was served as an internal control. Data shown are representative of a single experiment. PCR reactions were repeated at least twice

SFN treatment of HT-29 cells were investigated. As shown in Fig. 2b, cyclin D1, c-myc and cyclin A gene expression were strongly downregulated as early as 4 h after SNF treatment. Interestingly, cyclin E gene also showed a decrease at 4 h as compared with the control at that time point, but with the increasing time, the cyclin E gene expression in the control decreased significantly which resulted in a relative increase of cyclin E gene expression after 20 h. The gene expression of the cell cycle inhibitor $p21^{CIP1}$ increased slightly following 4 h and more significantly following 8 h of SFN treatment. The sustained strong downregulation of cyclin D1, cyclin A, and c-myc and upregulation of $p21^{CIP1}$, which were induced following SFN treatment, is consistent with the above-described G_1 cell cycle arrest.

SFN treatment decreases the expression of cyclin D1 and cyclin A but induces the expression of $p21^{CIP1}$

To determine if the SFN-induced alterations in transcription described above correlates with alterations in protein expression, we used Western blot analysis to examine the cellular protein levels of the G_1 cell cycle control genes cyclin D1, cyclin A, and $p21^{CIP1}$ following SFN treatment. Serum-starved HT-29 cells treated with SFN for 12 h displayed a moderate decrease in cyclin D1 protein levels at 25 μM and a dramatic decrease at 50 μM and 100 μM (Fig. 3a). Similarly, cyclin A protein levels significantly decreased only following treatment with high concentrations (> 50 μM) of SFN. In contrast, the cell cycle inhibitor $p21^{CIP1}$ protein levels were markedly induced with 25 μM and strongly induced with 50 μM SFN treatment. Interestingly, the protein levels of $p21^{CIP1}$ were unaffected with 100 μM SFN treatment; this effect may be a result of the strong cytotoxic effects of SFN at that high concentration. Because 50 μM of SFN had the strongest effect on cyclin D, cyclin A, and $p21^{CIP1}$ gene expression, we analyzed the expression of these genes over time following treatment of serum-starved HT-29 cells with 50 μM SFN. As shown in Fig. 3b, the expression of cyclin D1 was almost depleted following only 4 h of treatment. The expression of cyclin A was also strongly inhibited following SFN treatment and was totally depleted after 20 h. In contrast, the protein levels of $p21^{CIP1}$ were induced markedly after 4 h treatment and were dramatically induced thereafter. The SFN-induced alterations in protein expression of these three genes following 50 μM treatment persisted until 24 h, an effect that is consistent with the above-described cell cycle analysis and RT-PCR data.

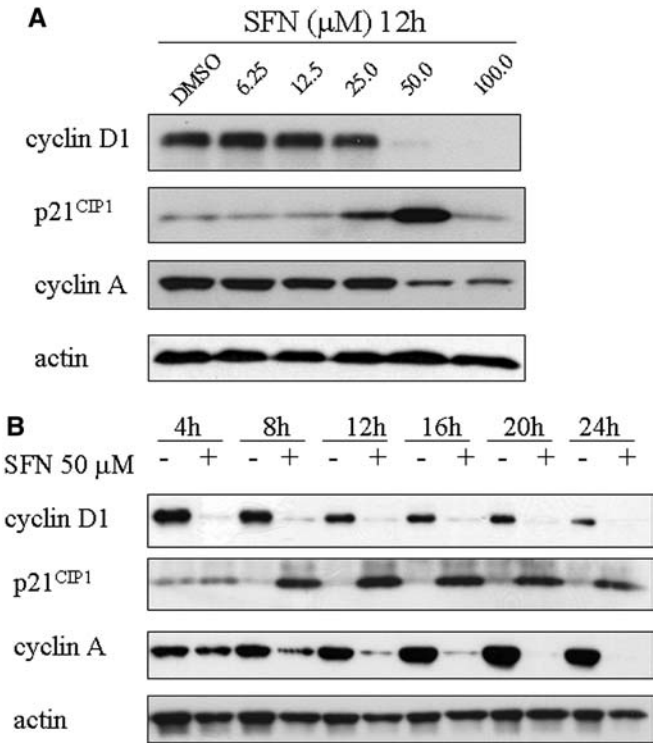


Fig. 3 Effects of treating HT-29 cells with SFN on the expression of various cell cycle regulatory proteins. After 36 h of serum-starvation, HT-29 cells were cultured in complete MEM medium and treated with (**a**) different doses of SFN for 12 h or (**b**) with 50 μM SFN for different periods of time (4, 8, 12, 16, 20 and 24 h). Control cells were treated with vehicle only (0.1% DMSO). Cell extracts were examined for cyclin D1, $p21^{CIP1}$, and cyclin A protein levels by Western blot analysis using the respective antibodies, as described in "Methods". Actin was used as an internal control. Data shown are representative of three independent experiments

SFN treatment activates MAP kinases

The signaling cascades associated with mitogen-induced MAPKs, such as ERK, JNK, and p38, are involved in cell proliferation, cell survival, growth inhibition, cell cycle arrest and apoptosis. Recent studies have shown that the expression of cyclin D1 and the p53-independent induction of $p21^{CIP1}$ may be regulated by MAPK pathways. Given the above-described SFN-induced alterations in cyclin D1 and $p21^{CIP1}$ gene expression, we next examined the effects of SFN treatment on MAP kinases. As shown in Fig. 4, at high concentrations (50 μM and 100 μM) of SFN, the MAPKs ERK, JNK, and p38 were all activated after only 4 h of treatment. It is somewhat contradictory that both the mitogen-induced ERK, which is associated with cell proliferation and survival, and the stress-activated JNK and p38, which are associated with growth inhibition and apoptosis, are activated by SFN. However, we propose that at high concentrations of SFN, the overall extent and the kinetics of the activation of the three different pathways as well as the activities of other signaling pathways, such as NF-κB, would determine the final fate of cells. In this study, the overall growth inhibitory effects associated with the activation of JNK and p38 may overcome the survival effects associated with ERK activation. The end result would favor cell growth inhibition.

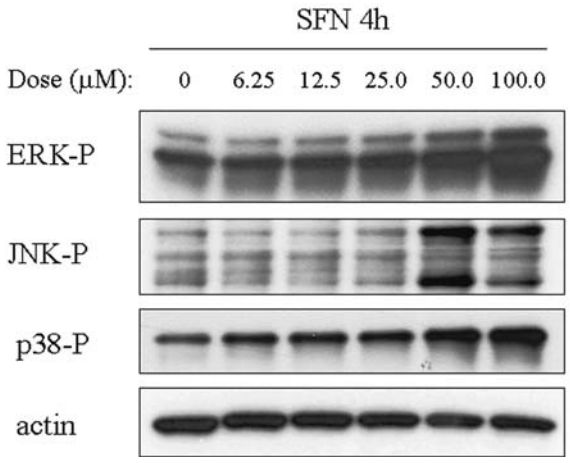


Fig. 4 MAP Kinases activation by SFN. HT-29 cells were serum starved for 36 h, then incubated in complete MEM medium containing vehicle or varying doses of SFN. After 4 h, cells were harvested and lysates were prepared. Western blot analysis for MAP kinase activation was performed as described in "Methods". Phosphorylation of ERK, JNK, and p38 were detected by immunoblotting with respective phosphor-specific antibodies. Actin was served as an internal control. Data shown are representative of two independent experiments

Antioxidant NAC and GSH block the effects of SFN

As a cancer chemoprevention agent, SFN can function not only as an antioxidant but also as a pro-oxidant. It has been proposed that the oxidative stress induced by SFN can lead to the activation of a wide variety of cellular signaling cascades, such as activation of MAP kinases, which would result in the induction of antioxidant response element (ARE)-mediated gene transcription. To determine if the SFN-mediated effects on cell cycle arrest, cell cycle regulated gene expression, and MAP kinase activation were associated with its ability to induce oxidative stress, serum-starved HT-29 cells were treated with both SFN (50 μM) and an antioxidant (either GSH or NAC) in MEM medium containing 10% FBS. As shown in Fig. 5a, treatment with SFN and NAC (1 or 5 mM) for 4 h completely blocked the inhibition of cyclin D1 expression and the induction of $p21^{CIP1}$ expression that is observed with SFN treatment alone. Treatment with both SFN (50 μM) and GSH (5 mM) also completely abolished the SFN-mediated inhibition of cyclin D1 expression, but only partially blocked the SFN-mediated induction of $p21^{CIP1}$ expression. Additionally, treatment with both SFN and either antioxidant also blocked the SFN-mediated activation of the MAP kinases ERK, JNK and p38 (Fig. 5b). Finally, treatment with SFN (50 mM) and either NAC (1 and 5 mM) or GSH (5 mM) significantly blocked the SFN-induced G_1 cell cycle arrest observed following 12 h exposure (Fig. 5c). These results suggest that the oxidative stress induced by SFN treatment may play a role in SFN-mediated G_1 cell cycle arrest, regulation of cell cycle-associated gene expression, and activation of MAPKs.

Role of MAP kinases-specific inhibitors on SFN effects

To further examine the role of MAP kinase activation in SFN-mediate G_1 cell cycle arrest, serum-starved HT-29 cells were pretreated with specific MAP kinase inhibitors for 30 min in MEM medium containing 10% FBS, then treated with 50 μM SFN. As shown in Fig. 6a, pre-treatment with either the ERK pathway-specific inhibitor PD98059 (25 μM) or the p38 pathway-specific inhibitor SB203580 (10 μM) completely abrogated the induction of $p21^{CIP1}$ expression induced by SFN treatment after 4 h, but enhanced the SFN-mediated inhibition of cyclin D1 expression. In contrast, while pre-treatment with the JNK pathway-specific inhibitor SP600125 (10 μM) prior to SFN treatment enhanced the SFN-mediated induction of $p21^{CIP1}$, whereas it only partially blocked the SFN-mediated inhibition of cyclin D1 expression. These results demonstrate that the activation of ERK and p38 pathways play similar roles in the altered regulation of $p21^{CIP1}$ and cyclin D1 expression in response to SFN treatment in HT-29 cells, but that role is different from the one played by JNK. SFN-mediated activation of the JNK pathway in HT-29 cells leads to an induction of $p21^{CIP1}$ expression and an inhibition of cyclin D1 expression; however, the effect of the JNK pathway on the SFN-mediated inhibition of cyclin D1 expression may not be its primary function, since blocking the activation of JNK only partially abrogated the inhibition of cyclin D1 expression induced by SFN. Because cyclin D1 and $p21^{CIP1}$ have antagonizing functions on G_1 cell cycle regulation, these results suggest that all the three MAP kinases have differential roles in controlling G_1 cell cycle progression. Analysis of cell cycle progression of cells pretreated with the specific MAP kinases inhibitor followed by 12 h SFN (50 μM) treatment revealed that the kinase inhibitors did not alter the ability of SFN to mediated a G_1 cell cycle arrest (Fig. 6b, c). This result is not unexpected, given that all the three MAP kinase specific inhibitors have differential effects on the SFN-induced $p21^{CIP1}$ induction and cyclin D1 inhibition.

Discussion

The present study demonstrates that treating HT-29 cells with high concentration of SFN inhibits the re-initiation of cell growth induced by serum. Previously, Gamet-Payrastre et al. [19, 22, 30] reported that SFN induced a G_2/M cell cycle arrest in HT-29 cells and inhibit quiescent HT-29 cells to re-enter the cell cycle. Interestingly, in SFN-induced G_2/M arrest study, they also showed that SFN induced a 15% increase of G_1 phase and only a 5% increase of G_2 phase after 24 h treatment. The G2/M cell cycle arrest effect was demonstrated after longer treatment (48 h and 72 h). Our results from using G_0/G_1 phase synchronized HT-29 cells are consistent with their earlier study in which

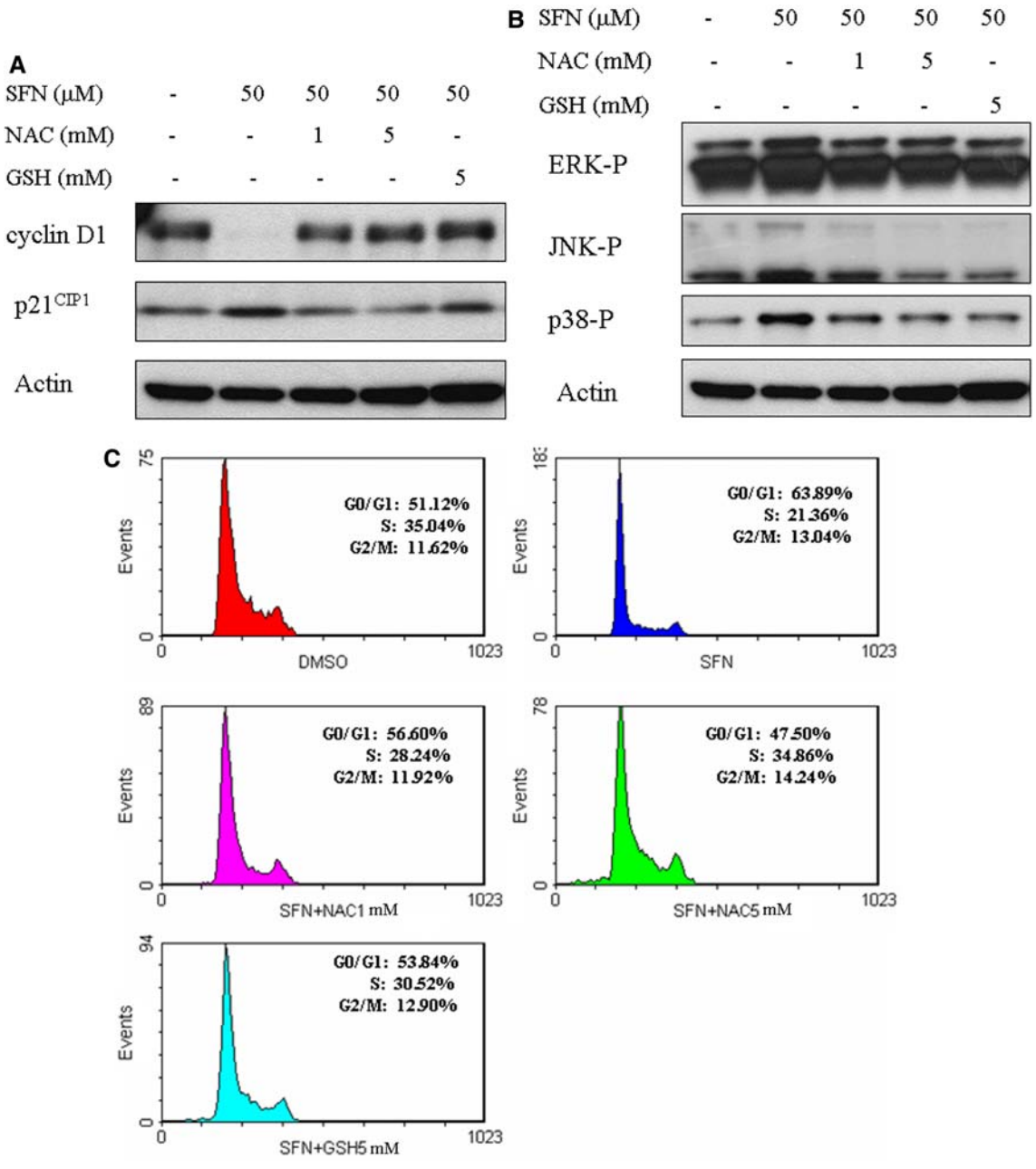
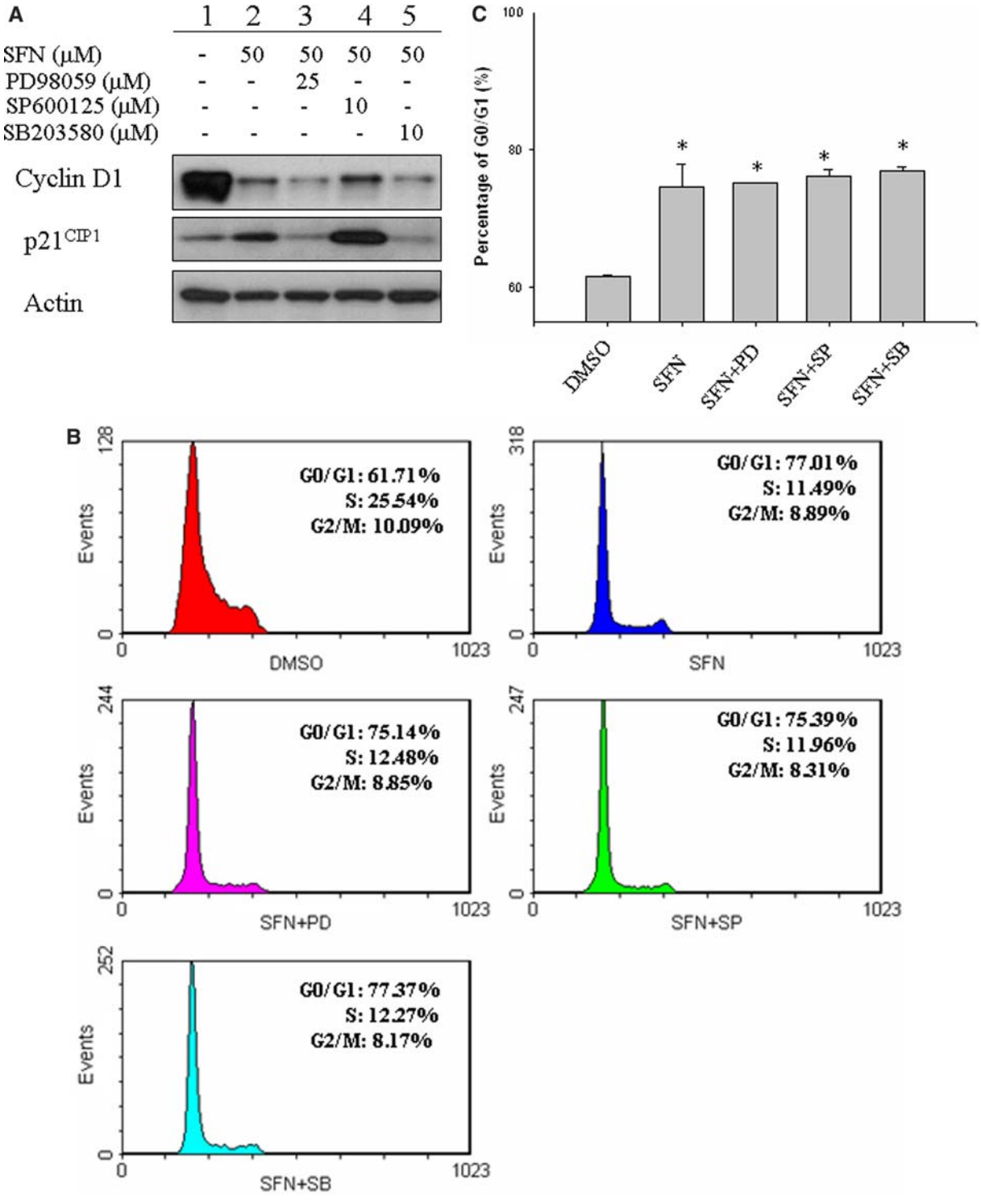


Fig. 5 Effects of the antioxidants NAC and GSH on the alteration of cell cycle regulatory protein expression, MAP kinases activation and G_1 cell cycle arrest by SFN. HT-29 cells cultured in six-well plates were first serum-starved for 36 h, then grown in complete MEM medium containing 50 μM SFN alone or in combination with either the antioxidant NAC (1 and 5 mM) or GSH (5 mM) for 4 h. The cells were then harvested and western blot analysis of (**a**) cell cycle regulatory protein expression and (**b**) activated MAPK kinases were performed as described in "Methods". Actin was served as an internal control. Data shown are representative of two independent experiments. (**c**) HT-29 cells cultured in 60-mm petri dish were serum-starved for 36 h, then grown in complete MEM medium containing 50 μM SFN alone or in combination with either the antioxidant NAC (1 and 5 mM) or GSH (5 mM) for 12 h. The cells were harvested and their cell cycle distribution was analyzed as described in "Methods.

reinitation of cell cycle were blocked by SFN treatment under serum stimulation condition, although the experimental conditions are not exactly the same. Nevertheless, we clearly demonstrate that SFN, at high concentrations, block the cell cycle in G_1 phase under serum-stimulation condition after synchronizing HT-29 cells, and that this arrest is accompanied by the downregulation of cell proliferation-related c-myc expression, the downregulation of positive cell cycle regulators such as cyclin D1, cyclin A and cyclin E, and the upregulation of negative cell cycle regulators such as the cyclin-dependent kinase inhibitor $p21^{CIP1}$.

Cell cycle progression is mediated by various cyclin-dependent kinases (cdk) whose activities are regulated by cyclin-dependent kinase inhibitors, such as $p21^{CIP1}$



and $p27^{KIP1}$ [31–34]. During a G_1 phase cell cycle arrest, cyclin-dependent kinase inhibitors bind to cdk/cyclin complexes (i.e. CDK4,6/cyclin D), thereby inhibiting their kinase activity on retinoblastoma (Rb) protein. Hypophosphorylated Rb sequesters the nuclear transcription factor E2F in the cytoplasm, resulting in an inhibition of expression of E2F-regulated genes that are critical for the transition from G_1 phase to S phase [34, 35]. In the present study, the induction of $p21^{CIP1}$ expression and the inhibition of cyclin D1, cyclin A and cyclin E expression demonstrates that SFN mediates its growth inhibitory effect via G_1 phase cell cycle arrest in serum-stimulated HT-29 cells.

MAPK cascades, such as the ERK, JNK and p38 pathways, are involved in a wide variety of cellular events such as proliferation, differentiation, growth arrest, and apoptosis. These pathways can be quickly activated in response to cellular oxidative stress generated

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Fig. 6 Effects of inhibiting MAP kinase pathways on SFN-mediated G_1 cell cycle regulatory protein expression and SFN-induced G_1 phase cell cycle arrest. **a** HT-29 cells in six-well plates were serum-starved for 36 h, and then grown in complete MEM medium containing either vehicle control (0.1 % DMSO) or a specific MAP kinase inhibitor as indicated in the figure. After 30 min, cells were exposed to 50 μM of SFN for an additional 4 h. The cells were harvested and western blot analysis of cyclin D1 and $p21^{CIP1}$ were performed as described in "Methods". Data shown are representative of three independent experiments. **b** After 30 min of MAP kinase inhibitor pre-treatment, cells were treated with 50 μM of SFN for an additional 12 h. The cells were then harvested and cell cycle analysis was performed as described in "Methods". Data shown are representative of two independent experiments. **c** Statistical analysis of the percentage of cells in G_1 phase of the cell cycle based on the results of two independent experiments co-treated with MAP kinase inhibitors and 50 μM SFN. $*P < 0.05$

by chemopreventive agents [36]. Moreover, in vitro studies have demonstrated that MAPK pathways can regulate the expression of both cyclin D1 and $p21^{CIP1}$ in various cell types, although their effects are highly dependent on the cell type and on the magnitude or duration of the stimulus [37–40]. Our results indicate that SFN, at high concentrations, activated all the three MAPK pathways at 4 h after treatment. The intensity of activation was stronger at an earlier time point (2 h, data not shown). The activation of MAPKs is consistent with our previous work which demonstrated that SFN can activate MAPKs in HepG2 cells albeit to different intensities [41–43]. Interestingly, this SFN-mediated activation of MAPKs could be completely blocked by the antioxidants NAC or GSH in this study. Because many cancer chemopreventive agents, including SFN, have been shown to generate mild oxidative stress via activation of signaling pathways to enhance their cellular protection function [17, 44], this result suggests that SFN treatment may generate oxidative stress which activate the MAPKs pathways. In support of this finding, cotreatment of antioxidant NAC and GSH with SFN, which block the SFN-mediated activation of MAPKs pathways, also block the induction of $p21^{CIP1}$ expression and inhibition of cyclin D1 expression as well as the G_1 phase cell cycle arrest. These results demonstrate an important role of the activation of MAPKs in the induction of $p21^{CIP1}$ expression, the inhibition of cyclin D1 expression, and the G_1 phase cell cycle arrest induced by SFN treatment.

Specific MAPKs inhibitors were used to further examine the role of MAPKs in the SFN-mediated alterations in the expression of cyclin D1 and $p21^{CIP1}$. Since both MAPKs and β-catenin/TCF signaling pathways are well-known pathways of regulating cyclin D1 gene expression [28, 37, 38, 40, 45, 46], SFN effects on both pathways were examined; nevertheless, SFN treatment did not significantly alter the whole cell or nuclear levels of β-catenin (data not shown) in HT-29 cells. This finding led us to focus more on MAPKs pathways in the SFN-mediated regulation of cyclin D1 expression. The use of ERK and p38 pathway-specific inhibitors demonstrated that the SFN-mediated activation of these pathways leads to the upregulation of cyclin D1 expression; in contrast, the SFN-mediated activation of the JNK pathway results in the downregulation of cyclin D1 expression. It should be noted that blocking the SNF-mediated JNK activation did not completely rescue the inhibition of cyclin D1 expression, suggesting that other signaling pathways might also be involved in the SFN-induced downregulation of cyclin D1. One potential candidate is the NF-κB signaling pathway. Several previous studies have shown that cyclin D1 is also a target of NF-κB signaling pathway and that the promoter region of cyclin D1 contains a NF-κB binding site [47–49]. Additionally, we have previously demonstrated that in a HT-29 cell line stably transfected with a NF-κB reporter gene, SFN could inhibit lipopolysaccharide-induced NF-κB activity in a dose-dependent manner [50]. Therefore, we propose that the SFN-mediated inhibition of cyclin D1 expression likely also involves the inhibition of NF-κB activity. Additional studies are required to demonstrate this effect.

The expression of $p21^{CIP1}$ is regulated through both p53-dependent and p53-independent mechanisms [51]. Activation of MAPK pathways have been implicated in the p53-independent regulation of $p21^{CIP1}$ expression. In the present study, activation of both the ERK and p38 pathways by SFN treatment induced the expression of $p21^{CIP1}$. In contrast, activation of the JNK pathway by SFN inhibited the expression of $p21^{CIP1}$. In summary, the SFN-mediated activation of ERK and p38 pathways in HT-29 cells induces the expression of both cyclin D1 and $p21^{CIP1}$, while the activation of the JNK pathway inhibits the expression of both cyclin D1 and $p21^{CIP1}$. Because cyclin D1 and $p21^{CIP1}$ have opposing regulatory functions in G_1 phase cell cycle control, the activation of these MAPK pathways by SFN has differential effects on G_1 phase cell cycle progression. This may indicate a natural feedback mechanism of regulating signaling pathways. In support of this finding, specific MAPK pathway inhibitors were unable to block the SFN-mediated G_1 cell cycle arrest.

In summary, SFN inhibits the serum-stimulated growth of HT-29 cells by blocking the cell cycle at G_1 phase. This arrest is accompanied with the upregulation of $p21^{CIP1}$ expression and the downregulation of cyclin D1, cyclin A, cyclin E, and c-myc expression. Although these events might be a result of SFN-induced oxidative stress, and activation of MAPK pathways was involved in the regulation of cyclin D1 and $p21^{CIP1}$ in response to SFN in HT-29 cells, our results indicated that SNF-mediated activation of MAPK pathways does not appear to have an exclusive role in controlling the G_1 cell cycle progression; the involvement of other signaling pathways such as NF-κB need to be further investigated.

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References

1. Potter JD, Slattery ML, Bostick RM, Gapstur SM (1993) Colon cancer: a review of the epidemiology. Epidemiol Rev 15:499–545
2. Steinmetzand KA, Potter JD (1991) Vegetables, fruit, and cancer. I. Epidemiology. Cancer Causes Control 2:325–357
3. Leoni O, Iori R, Palmieri S (2000) Hydrolysis of glucosinolates using nylon-immobilized myrosinase to produce pure bioactive molecules. Biotechnol Bioeng 68:660–664
4. Getahunand SM, Chung FL (1999) Conversion of glucosinolates to isothiocyanates in humans after ingestion of cooked watercress. Cancer Epidemiol Biomarkers Prev 8:447–451
5. Hecht SS, Kenney PM, Wang M, Upadhyaya P (2002) Benzyl isothiocyanate: an effective inhibitor of polycyclic aromatic hydrocarbon tumorigenesis in A/J mouse lung. Cancer Lett 187:87–94
6. Zhang Y, Kensler TW, Cho CG, Posner GH, Talalay P (1994) Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. Proc Natl Acad Sci USA 91:3147–3150
7. Singletaryand K, MacDonald C (2000) Inhibition of benzo[a]pyrene- and 1,6-dinitropyrene-DNA adduct formation in human mammary epithelial cells bydibenzoylmethane and sulforaphane. Cancer Lett 155:47–54
8. Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, Lozniewski A (2002) Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. Proc Natl Acad Sci USA 99:7610–7615
9. Kassie F, Uhl M, Rabot S, Grasl-Kraupp B, Verkerk R, Kundi M, Chabicovsky M, Schulte-Hermann R, Knasmuller S (2003) Chemoprevention of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)-induced colonic and hepatic preneoplastic lesions in the F344 rat by cruciferous vegetables administered simultaneously with the carcinogen. Carcinogenesis 24:255–261
10. Chung FL, Conaway CC, Rao CV, Reddy BS (2000) Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate. Carcinogenesis 21:2287–2291
11. Verhoeven DT, Verhagen H, Goldbohm RA, van den Brandt PA, van Poppel G (1997) A review of mechanisms underlying anticarcinogenicity by brassica vegetables. Chem Biol Interact 103:79–129
12. Faheyand JW, Talalay P (1999) Antioxidant functions of sulforaphane: a potent inducer of phase II detoxication enzymes. Food Chem Toxicol 37:973–979
13. Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S (2002) Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res 62:5196–5203
14. Langouet S, Furge LL, Kerriguy N, Nakamura K, Guillouzo A, Guengerich FP (2000) Inhibition of human cytochrome P450 enzymes by 1,2-dithiole-3-thione, oltipraz and cits derivatives, and sulforaphane. Chem Res Toxicol 13:245–252
15. Maheo K, Morel F, Langouet S, Kramer H, Le Ferrec E, Ketterer B, Guillouzo A (1997) Inhibition of cytochromes P-450 and induction of glutathione S-transferases by sulforaphane in primary human and rat hepatocytes. Cancer Res 57:3649–3652
16. Chen YR, Wang W, Kong AN, Tan TH (1998) Molecular mechanisms of c-Jun N-terminal kinase-mediated apoptosis induced by anticarcinogenic isothiocyanates. J Biol Chem 273:1769–1775
17. Payen L, Courtois A, Loewert M, Guillouzo A, Fardel O (2001) Reactive oxygen species-related induction of multidrug resistance-associated protein 2 expression in primary hepatocytes exposed to sulforaphane. Biochem Biophys Res Commun 282:257–263
18. Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C (2001) Nuclear factor kappa B is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem 276:32008–32015
19. Gamet-Payrastre L, Li P, Lumeau S, Cassar G, Dupont MA, Chevolleau S, Gasc N, Tulliez J, Terce F (2000) Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res 60:1426–1433
20. Pham NA, Jacobberger JW, Schimmer AD, Cao P, Gronda M, Hedley DW (2004) The dietary isothiocyanate sulforaphane targets pathways of apoptosis, cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. Mol Cancer Ther 3:1239–1248
21. Chiao JW, Chung FL, Kancherla R, Ahmed T, Mittelman A, Conaway CC (2002) Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. Int J Oncol 20:631–636
22. Parnaud G, Li P, Cassar G, Rouimi P, Tulliez J, Combaret L, Gamet-Payrastre L (2004) Mechanism of sulforaphane-induced cell cycle arrest and apoptosis in human colon cancer cells. Nutr Cancer 48:198–206
23. Haristoy X, Angioi-Duprez K, Duprez A, Lozniewski A (2003) Efficacy of sulforaphane in eradicating Helicobacter pylori in human gastric xenografts implanted in nude mice. Antimicrob Agents Chemother 47:3982–3984
24. Singh AV, Xiao D, Lew KL, Dhir R, Singh SV (2004) Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts in vivo. Carcinogenesis 25:83–90
25. Johnsonand DG, Walker CL (1999) Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol 39:295–312
26. Weinstein IB (2000) Disorders in cell circuitry during multistage carcinogenesis: the role of homeostasis. Carcinogenesis 21:857–64
27. Chan-Huiand PY, Weaver R (1998) Human mitogen-activated protein kinase kinase kinase mediates the stress-induced activation of mitogen-activated protein kinase cascades. Biochem J 336(Pt 3):599–609
28. Terada Y, Inoshita S, Nakashima O, Kuwahara M, Sasaki S, Marumo F (1999) Regulation of cyclin D1 expression and cell cycle progression by mitogen-activated protein kinase cascade. Kidney Int 56:1258–1261
29. Kerkhoffand E, Rapp UR (1998) Cell cycle targets of Ras/Raf signalling. Oncogene 17:1457–1462
30. Gamet-Payrastre L, Lumeau S, Gasc N, Cassar G, Rollin P, Tulliez J (1998) Selective cytostatic and cytotoxic effects of glucosinolates hydrolysis products on human colon cancer cells in vitro. Anticancer Drugs 9:141–148
31. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D (1993) p21 is a universal inhibitor of cyclin kinases. Nature 366: 701–704
32. Toyoshimaand H, Hunter T (1994) p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 78:67–74
33. Granaand X, Reddy EP (1995) Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11:211–219
34. Hofseth LJ, Hussain SP, Harris CC (2004) p53: 25 years after its discovery. Trends Pharmacol Sci 25:177–181
35. Paggi MG, Baldi A, Bonetto F, Giordano A (1996) Retinoblastoma protein family in cell cycle and cancer: a review. J Cell Biochem 62:418–430
36. Chen C, Shen G, Hebbar V, Hu R, Owuor ED, Kong AN (2003) Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. Carcinogenesis 24:1369–1378
37. Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A, Pestell RG (1995) Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J Biol Chem 270:23589–23597

38. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J (1996) Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem 271:20608–20616
39. Rooversand K, Assoian RK (2000) Integrating the MAP kinase signal into the G1 phase cell cycle machinery. Bioessays 22:818–826
40. Oh SY, Park KS, Kim JA, Choi KY (2002) Differential modulation of zinc-stimulated p21(Cip/WAF1) and cyclin D1 induction by inhibition of PI3 kinase in HT-29 colorectal cancer cells. Exp Mol Med 34:27–31
41. Yu R, Lei W, Mandlekar S, Weber MJ, Der CJ, Wu J, Kong AT (1999) Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. J Biol Chem 274: 27545–27552
42. Yu R, Mandlekar S, Lei W, Fahl WE, Tan TH, Kong AT (2000) p38 mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens. J Biol Chem 275:2322–2327
43. Kim BR, Hu R, Keum YS, Hebbar V, Shen G, Nair SS, Kong AN (2003) Effects of glutathione on antioxidant response element-mediated gene expression and apoptosis elicited by sulforaphane. Cancer Res 63:7520–7525
44. Paolini M, Perocco P, Canistro D, Valgimigli L, Pedulli GF, Iori R, Croce CD, Cantelli-Forti G, Legator MS, Abdel-Rahman SZ (2004) Induction of cytochrome P450, generation of oxidative stress and in vitro cell-transforming and DNA-damaging activities by glucoraphanin, the bioprecursor of the chemopreventive agent sulforaphane found in broccoli. Carcinogenesis 25:61–67
45. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 96:5522–5527
46. Tetsuand O, McCormick F (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398:422–426
47. Baldwin AS Jr, Azizkhan JC, Jensen DE, Beg AA, Coodly LR (1991) Induction of NF-kappa B DNA-binding activity during the G0-to-G1 transition in mouse fibroblasts. Mol Cell Biol 11:4943–4951
48. Joyce D, Bouzahzah B, Fu M, Albanese C, M. D'Amico, Steer J, Klein JU, Lee RJ, Segall JE, Westwick JK, Der CJ, Pestell RG (1999) Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor-kappaB-dependent pathway. J Biol Chem 274:25245–25249
49. Suzui M, Masuda M, Lim JT, Albanese C, Pestell RG, Weinstein IB (2002) Growth inhibition of human hepatoma cells by acyclic retinoid is associated with induction of p21(CIP1) and inhibition of expression of cyclin D1. Cancer Res 62:3997–4006
50. Jeong WS, Kim IW, Hu R, Kong AN (2004) Modulatory properties of various natural chemopreventive agents on the activation of NF-kappaB signaling pathway. Pharm Res 21:661–670
51. Garteland AL, Tyner AL (1998) The growth-regulatory role of p21 (WAF1/CIP1). Prog Mol Subcell Biol 20:43–71